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RESEARCH ARTICLE

Obligate Brood Parasites Show More Functionally Effective Innate Immune Responses: An Eco-immunological Hypothesis

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Abstract Immune adaptations of obligate brood parasites attracted interest when three New World cowbird species (Passeriformes, Icteridae, genus *Molothrus*) proved unusually resistant to West Nile virus. We have used cowbirds as models to investigate the eco-immunological hypothesis that species in parasite-rich environments characteristically have enhanced immunity as a life history adaptation. As part of an ongoing program to understand the cowbird immune system, in this study we measured degranulation and oxidative burst, two fundamental responses of the innate immune system. Innate immunity provides non-specific, fast-acting defenses against a variety of invading pathogens, and we hypothesized that innate immunity experiences particularly strong selection in cowbirds, because their life history strategy exposes them to diverse novel and unpredictable parasites. We compared the relative effectiveness of degranulation and oxidative burst responses in two cowbird species and one related, non-parasitic species. Both innate immune defenses were significantly more functionally efficient in the two parasitic cowbird species than in the non-parasitic red-winged blackbird (Icteridae, *Agelaius phoeniceus*). Additionally, both immune defenses were more functionally efficient in the brown-headed cowbird (*M. ater*), an extreme host-generalist brood parasite, than in the bronzed cowbird (*M. aeneus*), a moderate host-specialist with lower exposure to other species and their parasites. Thus the relative effectiveness of these two innate immune responses corresponds to the diversity of parasites in the niche of each species and to their relative resistance to WNV. This study is the first use of these two specialized assays in a comparative immunology study of wild avian species.

Keywords Host-parasite coevolution · Brood parasite · Cowbird · Degranulation · Eco-immunology · Parasite-mediated selection · Innate immunity · Oxidative burst

Introduction

Design and functionality of the immune system is increasingly recognized to play an important role in species fitness (Schulenburg et al. 2009; Schmid-Hempel 2011). It is expected that each species has a customized "portfolio" of immune defenses that reflects its adaptations to its environment and life history strategy (Schmid-Hempel 2003). Immune adaptations of obligate brood parasites attracted interest when several New World cowbird species were found to be unusually resistant to West Nile virus (Flaviviridae, *Flavivirus*, WNV), a non-native, highly virulent pathogen (Reisen and Hahn 2007; Hahn and Reisen 2011). Cowbird viremia levels post-infection with WNV were among the lowest when compared to many other species (Komar et al. 2003; Reisen et al. 2003), even though North American songbirds (Passeriformes) were particularly hard hit by WNV (Kilpatrick et al. 2007; Hofmeister 2011). All species less susceptible than cowbirds to WNV were in orders that were taxonomically very distant from songbirds, such as

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Psittaciformes (parakeets), Galliformes (quail, chickens, pheasants) and Columbiformes (rock doves) (Kilpatrick et al. 2007; Reisen and Hahn 2007).

Examination of the cowbird portfolio of immune defenses promised insights into the design of an unusually effective immune system. We have previously reported experimental infection studies in which several cowbird species had greater resistance to endemic arboviruses (WEEV,[1] SLEV,[2] and a challenging co-infection[3]) as well as WNV, and in a comparative study of maternal investment we reported significantly higher levels of immune elements in cowbird eggs (Hahn et al. 2012). In this study we focused on innate immunity, since these non-specific, fast-acting defenses likely experience strong selection in obligate brood parasites like cowbirds, which regularly encounter novel and unpredictable parasites[4] due to their unusual life history strategy (Ortega 1998; Davies 2000). Female cowbirds come into contact with ectoparasites in nests of other species when they lay their eggs, and cowbird nestlings have prolonged physical contact with and exposure to their foster parent species which provide warmth post-hatching (Ortega 1998; Davies 2000), then feed them by mouth for approximately 30 days (Hahn and Fleischer 1995; Hahn et al. 1999). Independent evidence of the higher parasite diversity of the cowbird niche was provided in our study of louse infections, which showed that both juvenile and adult brown-headed cowbirds (*M. ater*) are infected with significantly more diverse avian louse species than are songbird species that are not brood parasites (Hahn et al. 2000). Further exposure unique to brood parasites includes the species-specific helminths of their foster parent species (Rausch 1983) as well as their foreign salivary (Kyle and Kyle 1990) and gut microbes (Ley et al. 2008). The extent to which cowbirds are exposed to foreign microbes as a result of their unusual contacts with other species is increasingly confirmed as genomic sequencing studies reveal the species-specificity and tissue-specificity of microbial communities (Godoy-Vitorino et al. 2008; Ley et al. 2008).

An indication that innate immune mechanisms would likely show specialized adaptations in cowbirds was the superior resistance of cowbirds to the non-native WNV (Reisen and Hahn 2007), since innate immunity has the major role in identifying, controlling and eliminating novel pathogens like WNV (Janeway et al. 2001). Furthermore, the rapid rate of recovery in experimental infection studies with WNV (Komar et al. 2003; Reisen and Hahn 2007)—birds that do recover show a rapid drop in viremia levels within a few days of infection—is more characteristic of innate immunity than slower-acting adaptive immunity (Janeway et al. 2001).

As a first step in examining the cowbird innate immune system, we measured degranulation and oxidative burst, two fundamental components of phagocytosis, the process by which solid particles such as bacteria and other microbes are engulfed by the cell, killed, and degraded (Serbina et al. 2008). In oxidative burst, killing occurs by an oxygen-dependent process that uses NADPH[5] and produces reactive oxygen species (ROS) to destroy the engulfed material (Kogut et al. 2001, 2007; Sild and Hõrak 2010). In contrast, in degranulation killing occurs by an oxygen-independent process that depends on the release of granules to destroy or deprive the engulfed particles of material needed for growth (Hume 2006; Serbina et al. 2008). We chose these two immune mechanisms because they are specific functional killing mechanisms, one oxygen-dependent, one oxygen-independent, and because they are measured by assays that are highly reliable (Swaggerty et al. 2003).

We compared oxidative burst and degranulation responses in two cowbird species, brown-headed cowbird and bronzed cowbird, and one non-parasitic relative, the red-winged blackbird (Ellison and Lowther 2009). Red-winged blackbird is in the same taxonomic branch of the Family Icteridae as the cowbird (i.e. grackles and allies; Lanyon and Omland 1999) and is similar in many aspects of behavior and ecology except brood parasitism (Yasukawa and Searcy 1995). The red-winged blackbird lacks the intimate exposure to other species and their parasites, which cowbirds encounter in each breeding event, so we predicted that the innate immune defenses of the blackbird experience lower selection and are less functionally efficient than those in cowbird species.

Because the two cowbird species have differing degrees of exposure to foreign parasites, we also predicted that their innate immune responses would differ and correspond in functional effectiveness to their relative exposure to other species. The brown-headed cowbird has the greatest exposure to foreign parasites, since it is an extreme host-generalist that lays its eggs in the nests of more than 200 avian host species representing 19 different taxonomic families (Lowther 1993). The bronzed cowbird, a moderate host-specialist, is exposed to a lower diversity of foreign parasites, primarily those associated with its traditional hosts, 11 oriole species in a sister genus, *Icterus* (Ellison and Lowther 2009).

[1] Western equine encephalomyelitis virus (Togaviridae, *Alphavirus*, WEEV).

[2] St. Louis encephalitis virus (Flaviviridae, *Flavivirus*, SLEV).

[3] WNV and WEEV.

[4] "Parasite" is used here in its widest sense, including viruses, bacteria, protists, and eukaryotes (Schulenburg et al. 2009).

[5] NADPH = reduced NADP (Nicotinamide adenine dinucleotide phosphate).

Materials and Methods

Species

Brown-headed cowbird is a small seed-eating songbird, 17.5 cm long and 40–50 g (Lowther 1993), one of five species of obligate brood parasites that compose the New World genus *Molothrus* and exploit other avian species to incubate their eggs and raise their young (Friedmann et al. 1977; Rothstein and Robinson 1998). It has a continental-scale range across North America (Sauer et al. 2005). We worked with *M.a. obscurus*, a subspecies whose range is in the southwestern United States and along the Pacific Coast (Lowther 1993).

Bronzed cowbird is a Central American species with a range extending into Colombia, South America (Friedmann 1929), which began invading Texas and Arizona in the twentieth century, expanding the number of its avian host species (Kostecke et al. 2004). Slightly larger than the brown-headed cowbird (22.2 cm; 56–68 g), it is also a seed-eating species that feeds in grasslands, agricultural and edge areas (Ellison and Lowther 2009).

Red-winged blackbird is a seed-eating species, and its size (20.5 cm; 55–65 g) is similar to the bronzed cowbird (Ehrlich et al. 1988). It is similar in ecology, behavior, and breeding system to cowbird species except for the habit of brood parasitism (Yasukawa and Searcy 1995). Red-winged blackbirds nest in wet, brushy, and marshy areas as well as adjacent upland fields, and they feed in these habitats as well as in grasslands and agricultural areas (Yasukawa and Searcy 1995). Red-winged blackbird is one of the most abundant species in North America (Dolbeer 2003) and has a continental-scale range like the brown-headed cowbird (Sibley 2000).

Study Site and Fieldwork

The study site lies on the Edwards Plateau in Texas, a mixed ash-juniper and oak woodlands habitat with interspersed grasslands. The study was done in May 2009, on Fort Hood, Texas, in conjunction with a cowbird control project that protects breeding populations of two endangered species, golden cheek warbler (*Dendroica chrysoparia*) and black capped vireo (*Vireo atricapillus*) (Hayden et al. 2000). We collected 0.15 ml blood from each of 30 brown-headed cowbirds, 30 bronzed cowbirds, and 30 red-winged blackbirds that were trapped in large walk-in traps baited with white millet. Birds were trapped and handled under appropriate state and federal permits: Title 50, Code of Federal Regulations, part 21.43 (Depredation order for blackbirds, cowbirds, grackles, crows and magpies) and Title 50 CRF 21.12 (Permit for research by public institutions). Protocols were approved by Patuxent Wildlife Research Center Animal Care and Use Committee (ACUC) and USDA-ARS—Southern Plains Agricultural Research Center (ACUC).

Immune Response Assays

Whole blood assays that measure functional effectiveness of leukocytes were carried out within 2–3 h of blood collection at the USDA—Southern Plains Agricultural Research Center, College Station, TX. Methodology for assays to measure oxidative burst and degranulation is described in Kogut et al. (2001, 2007). The oxidative burst assay uses chemiluminescence to measure the release of toxic oxygen radicals made by the phagocytic leukocytes. The degranulation assay measures the release of a primary enzyme (β-D glucuronidase) that is stored in pre-formed granules in the host cytoplasm (Anderem and Underhill 1997).

Blood was collected from each bird and placed in plastic vacutainer tubes spray coated with K_2EDTA (disodium ethylenediaminetetraacetic acid) (BD vacutainer, Franklin Lakes, NJ), then tubes were shaken vigorously to prevent clotting and to mix thoroughly. For all 3 species, the blood from three separate groups of birds was combined, and independent assays were carried out using the three pools. Each pool was transferred to a conical tube, and 30 ml of 1 % methylcellulose prepared in RPMI 1640 medium was added, mixed thoroughly, and centrifuged at $35\times g$ for 20 min at 4 °C. The supernatant containing the peripheral blood leukocytes (PBL), including heterophils and monocytes, was transferred to a new conical tube and 10 ml clear RPMI (Roswell Park Memorial Institute medium) added; then cells were pelleted by centrifugation ($485\times g$ for 15 min at 4 °C). The cells were re-suspended (1×10^9/ml) in fresh RPMI (1 ml).

For each assay, prepared cells were equally divided into non-stimulated (controls) and stimulated cells.

1. Oxidative burst assay

Production of an oxidative burst by phorbol myristate (PMA)—stimulated peripheral blood leukocytes was measured for the two species by oxidation of DCFHDA (non-fluorescent 2′,7′-dichlorfluorescein-diacetate) to fluorescent DCF (2′,7′-dichlorfluorescein) as described previously (He et al. 2003) with modification. One milliliter of leukocytes (8×10^6 cells/ml) was added to 2-ml microcentrifuge tubes, then incubated with PMA (1.62 μM) and DCFH-DA (10 μg/ml in final concentration) for 1 h at 37 °C. The aliquots of cell cultures (150 μl) were then dispensed to black 96-well plate, and the fluorescence was measured using a GENios Plus Fluorescence Microplate Reader (TECAN US Inc., Research Triangle Park, NC) at 485 nm excitation and 530 nm emission wavelengths. The relative fluorescent units (RFU) were recorded after 60 min.

2. Degranulation assay

Steps (a) and (b) below were taken first, before the degranulation assay (c) was conducted.

(a) Bacterial preparation

A poultry isolate of *Salmonella enterica* serovar Enteritidis (*S. enteritidis*)(97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA, USA). *S. enteritidis* is a highly pathogenic bacterium that serves as a useful challenge pathogen in stimulating immune responses.

Salmonella enteritidis was cultured in tryptic soy broth (TSB; Difco Laboratories, Becton–Dickinson Co., Sparks, MD, USA) overnight at 41 °C. The bacteria were pelleted (7,700×*g*, 10 min), washed with cold phosphate-buffered saline (PBS) and centrifuged again (7,700×*g*, 10 min). The supernatant was discarded, and the pellet was resuspended in 1 ml cold PBS. A stock solution of *S. enteritidis* (1×10^9 colony forming units (CFU)/ml) was prepared in PBS. The bacterial concentration was determined spectrophotometrically (Spectronic 20D spectrophotometer; Milton Roy Co., Golden, CO, USA) using a standard curve with a reference wavelength of 625 nm. *S. enteritidis* were prepared fresh for each experiment and kept on ice until used.

(b) Opsonization of *S. enteritidis*

Opsonized *Salmonella enteritidis* (OpSE) was prepared as previously described (Ferro et al. 2005). Briefly SE (1×10^9 CFU/ml) were suspended in normal chicken serum (v:v [4:1]) and incubated for 60 min at 39 °C on a rotary shaker. OpSE was stored at 4 °C until used in the degranulation assays.

(c) Enzyme measurement

Degranulation was measured by quantifying β-D-glucuronidase activity (Kogut et al. 2001) in culture medium (RPMI without serum; 1 ml (8 × 106/ml) following stimulation of leukocytes (8×10^6/ml) with OpSE at 37 °C for 1 h on a rocker platform in 5 % CO_2 and 95 % humidity. After incubation, the cells were pelleted by centrifugation at 10,000×*g* for 2 min at 4 °C and supernatants were collected for the assay. An aliquot of 25 ml supernatant was incubated with 50 ml of freshly prepared substrate (10 mM 4-methylumbelliferyl-b-D-glucuronide and 0.1 % Triton X-100 in 0.1 M sodium acetate buffer) in a black 96-well plate for 4 h at 37 °C. The reaction was stopped by adding 200 ml of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4) to each well. Liberated 4-methylumbelliferone was measured fluorometrically (355/460 nm) using a fluorescence microplate reader (Genios Plus Plate Reader, TECAN US Inc).

Statistical Analysis

The data from the repeated experiments were combined for presentation and statistical analysis. Statistical differences between treatment groups were determined by ANOVA ($P < 0.05$). Means were further separated for significance with a pair-wise multiple comparison procedure (Tukey test, $P < 0.05$). Significant differences were further separated using Duncan's multiple range test.

Results

The oxidative burst assay, which measures toxic oxygen radicals available to kill bacteria, and the degranulation assay, which measures a primary enzyme available in host cytoplasmic granules, showed that phagocytic leukocytes of the three species were significantly different from one another (Figs. 1, 2). Responses of brown-headed cowbird and bronzed cowbird were both significantly more functionally efficient than those of the red-winged blackbird, and immune responses of the brown-headed cowbird were also significantly more effective than those of the bronzed cowbird.

Leukocyte Functional Assays

Oxidative Burst Assay

Leukocytes from brown-headed cowbirds, stimulated with PMA, produced a concentration-dependent oxidative burst (43,758 ± 540.77 RFU) that was significantly greater than

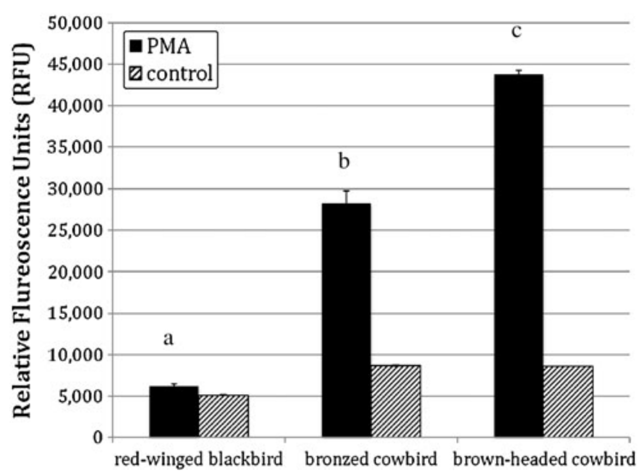

Fig. 1 A comparison of functional effectiveness of the oxidative burst response among three species. Cell source is total peripheral blood leukocytes. *PMA* phorbol myristate. Data presented as mean $\times 10^3$ ± standard error relative flourescence units (RFU). *Different letters* indicate significant differences at $P < 0.01$

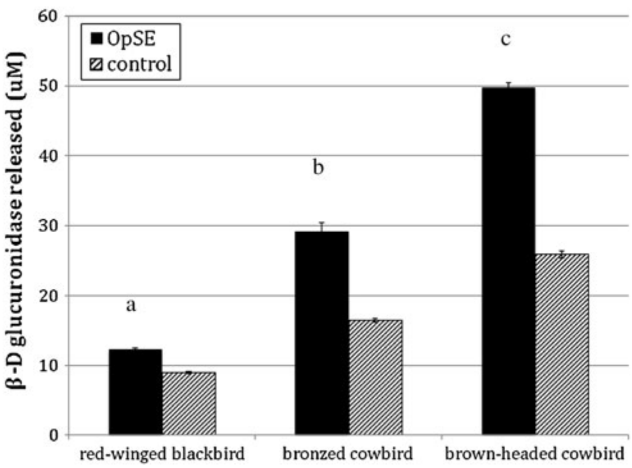

Fig. 2 A comparison of functional effectiveness of the degranulation response among the three species. Cell source is total peripheral blood leukocytes. *OpSE* opsonized *Salmonella enteritidis.* Data is presented as mean μM ± standard error of the mean of β-D-glucuronidase released. *Different letters* indicate significant differences at $P < 0.01$

that of leukocytes stimulated with PMA from bronzed cowbirds (28,305 ± 1,395.06 RFU), which in turn was significantly greater than the oxidative burst of red-winged blackbirds (6,257 ± 206 RFU)($P < 0.01$; Fig. 1). No significant differences were found between non-stimulated (control) leukocytes isolated from brown-headed cowbirds, bronzed cowbirds or red-winged blackbirds.

Degranulation Assay

Leukocytes from brown-headed cowbirds, stimulated with OpSE, induced a concentration-dependent release of the primary granule, β-D–glucuronidase, that was significantly greater (49.798 ± 0.661 μM) than that from leukocytes of bronzed cowbirds (29.105 ± 1.286 μM), which in turn was greater than that from leukocytes of red-winged blackbirds (12.326 μM ± 0.169)($P < 0.01$; Fig. 2).

Discussion

This study is part of our ongoing study of immune adaptations using the New World cowbirds, obligate brood parasites, as model organisms adapted to parasite-rich environments. We found that two cowbird species showed more effective innate immune responses, as measured by degranulation and oxidative burst assays, than the red-winged blackbird, a closely-related species that is not a brood parasite. Comparing the two cowbird species, the extreme host-generalist, brown-headed cowbird, showed more effective innate immune responses than the bronzed cowbird, a moderate host-specialist with lower exposure to other species and their parasites. Taken with our earlier studies showing that cowbirds have (1) greater resistance to infection with WNV and endemic arboviruses (Hahn and Reisen 2011), and (2) higher levels of immune components in cowbird eggs (Hahn et al. 2012), the results presented here contribute to an emerging portrait of enhanced immunity in the cowbirds.

We considered whether other factors besides brood parasitism, such as geographic range and mating system, could have affected the historic exposure patterns to pathogens of these species, but these factors do not differ in ways that correspond to the immune results. Regarding mating system, the 3 species studied are all polygamous (Ehrlich et al. 1988; Yasukawa and Searcy 1995; Jaramillo and Burke 1999). Regarding geographic range, brown-headed cowbird and red-winged blackbird have large, similar-sized ranges (Sauer et al. 2005), yet they are significantly different in the functional effectiveness of degranulation and oxidative burst responses. Bronzed cowbird's geographic range is much smaller than that of the other species (Ortega 1998), yet its immune responses are intermediate and significantly different from both.

This is the first study to report differences in functional effectiveness of immune responses between three closely related species for which there are also (1) quantitative measurements of environmental parasite richness (Hahn et al. 2000) and (2) experimental infection studies with virulent pathogens like WNV, WEEV, SLEV (Hahn and Reisen 2011). While several investigators have addressed the question whether parasite-rich environments favor the evolution of enhanced immunity (Piersma 1997; Blount et al. 2003; Lindstrom et al. 2004; Mendes et al. 2006), only recently have investigators (Buehler et al. 2009; Hawley and Altizer 2011; Horrocks et al. 2012) emphasized the importance of including measurements of environmental parasite diversity. Other life history and environmental parameters besides parasite diversity have been considered for their influence on the evolution of immunity, including (1) lifespan (Tella et al. 2002; Haussmann et al. 2005; Apanius and Nisbet 2006; Hahn and Smith 2011); (2) pace of life (Lee et al. 2006); (3) latitudinal gradient of breeding habitat (Ardia 2007; Lee et al. 2008); and (4) invasive character (Lee and Klasing 2004; Lee et al. 2005), and investigators will be challenged to disentangle the influences of these factors on the design of immune systems (Demas and Nelson 2012).

This is the first report of degranulation measured in a wild bird species so far as we know, and the assay described here demonstrates a particularly meaningful measure of this innate immune response, because it utilized a highly pathogenic challenge bacterium, *Salmonella enteritidis.* Our use of the oxidative burst assay is the first demonstration of differences among closely-related species in functional effectiveness of this immune defense. Papp and Smits

(2007) first demonstrated the use of the assay on wild species, carrying it out on five raptor and two waterfowl species although their purpose was not to make between-species comparisons. Sild and Hõrak (2010) used the assay on a single species to evaluate the effects of age and sex on oxidative burst response among individual greenfinches (*Carduelis chloris*). The oxidative burst assay promises to become a widely used tool in eco-immunology, because the immune response is an important one and the assay is highly reliable. The assay's usefulness will likely be greater if a single agent becomes the standard to measure chemiluminescence—in our assay, we used phorbol myristate (PMA); Sild and Hõrak (2010) used pholasin; and Papp and Smits (2007) used lucigenin—since comparisons cannot be made between studies that use different agents as light enhancer.

The innate immune assays used in this study reflect the increasing sophistication of eco-immunological studies in quantifying measurements of immunity (Millet et al. 2007; Demas et al. 2011). The degranulation and oxidative burst assays are highly repeatable measures of functional effectiveness of individual mechanisms (Swaggerty et al. 2003), they directly measure the toxic substances released by the study animal's immune cells (Kogut et al. 2001), and they permit reliable comparisons among species, which is encouraging for their future wide use on wild species (Tukel et al. 2006; Boyen et al. 2009). Degranulation and oxidative burst assays are also both highly specific measurements, while the widely-used bactericidal killing assay (BKA) provides a quantitative index of bacterial killing capacity based on the action of several immune elements in whole blood such as phagocytes, opsonizing proteins and natural antibodies. Merrill et al. (2013), using BKA, reported no difference in immune responses of cowbirds and red-winged blackbirds (genders combined) and lower responses in female cowbirds vs female red-winged blackbirds. Since BKA is less specific than the assays used in our study, it is not known which immune element(s) underlie their results. Further examination of particular immune elements would be useful as well as further examination of female immune responses to determine whether differences in their fecundity have an effect (Scott and Ankney 1983).

Our results encourage further investigation of cowbird immune defenses such as antiviral functional assays to see whether interferon production or antibody production by peripheral blood leukocytes have a role in viral infections, since innate immunity has both a signaling function to activate adaptive immune responses as well as an effector function (Janeway and Medzhitov 2002). While no single innate immune response can be assumed to predict all other innate immune responses (Adamo 2004; Matson et al. 2006), degranulation and oxidative burst are fundamental processes and represent a major element of innate immunity. Further examination of both innate and adaptive immune responses in cowbirds will be useful to understand the full range of their adaptations to their unusual niche. For example, we previously reported evidence that brown-headed cowbird produced significantly lower WNV antibody titres post-infection than three related, non-parasitic species (Reisen and Hahn 2007), which may indicate that cowbirds economize on this aspect of adaptive immunity.

The New World cowbirds provide an effective natural experiment for testing the hypothesis that ecological niches with heightened exposure to parasites favor the evolution of more effective immune responses (Moller 1997) for several reasons. First, all cowbird species are obligate brood parasites, and every cowbird breeding event entails exposure to foreign parasites and microbes. Other avian brood parasites, like ducks (Joyner 1976; Semela and Sherman 2001), shorebirds (Colwell 1986) and galliforms (Krakauer and Kimball 2009) are facultative parasites that experience inconsistent exposure to foreign parasites. Second, cowbirds parasitize only non-conspecifics, while many other avian brood parasites engage in intra-specific brood parasitism exclusively or occasionally and are not as frequently exposed to foreign parasites. Third, cowbirds are altricial, hatch without feathers or locomotor ability and experience prolonged physical intimacy with their foster parent species, while chicks of precocial species such as ducks, shorebirds, and galliform species do not. Fourth, cowbird species exemplify a range from host-generalists to host-specialists and thus offer a unique opportunity for comparative studies examining the effect on immunity of different degrees of exposure to parasites. The results of this study contribute to the emerging portrait of an unusual immune system in cowbirds.

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